U.S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE **TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 USC 371 AND 37 CFR 1.491**		ATTORNEY DOCKET NO. 215110 U.S APPLICATION NO. Unassigned 10/030464
INTERNATIONAL APPLICATION NO PCT/EP00/06539	INTERNATIONAL FILING DATE 10 JULY 2000 (10.07.00)	PRIORITY DATE CLAIMED 08 JULY 1999 (08.07.99)
TITLE OF INVENTION 11-ARACHIDONATE-LIPOXYGENASE MUTANTS		
APPLICANT(S) FOR DO/EO/US FEUSSNER, Ivo; HORNUNG, Ellen; ROSAHL, Sabine		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 USC 371 and 37 CFR 1.491.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371 and 37 CFR 1.491.

3. ☒ This is an express request to begin national examination procedures (35 USC 371(f)).

4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).

5. ☒ A copy of the International Application as filed (35 USC 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☒ An English language translation of the International Application as filed (35 USC 371(c)(2)).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 USC 371(c)(4)).

10. ☒ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).

11. Nucleotide and/or Amino Acid Sequence Submission
    a. ☐ Computer Readable Form (CRF)
    b. Specification Sequence Listing on:
       i. ☐ CD-ROM or CD-R (2 copies); or
       ii. ☐ Paper Copy
    c. ☐ Statement verifying identity of above copies

Items 12 to 19 below concern other document(s) or information included:

12. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
    ☐ Form PTO-1449
    ☐ Copies of Listed Documents

13. ☐ An assignment for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

14. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

15. ☐ A substitute specification.

16. ☐ A change of power of attorney and/or address letter.

17. ☒ Application Data Sheet Under 37 CFR 1.76

18. ☒ Return Receipt Postcard

19. ☒ Other items or information:
    Amendments to Specification, Claims, and Abstract Made Via Preliminary Amendment; Pending Claims After Entry of Preliminary Amendment

531 Rec'd PCT/ 08 JAN 2002

U.S. APPLICATION NO Unassigned 10/030464	INTERNATIONAL APPLICATION NO PCT/EP00/06539	ATTORNEY DOCKET NO. 215110

20. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1,040.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... $ 890.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO, but international search fee (37 CFR 1.445(a)(2))paid to USPTO ..... $ 740.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... $ 710.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1) to (4) ..... $ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT=	$890.00	
Surcharge of $130.00 for furnishing the National fee or oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	17 -20=	0	x $ 18.00	$0.00	
Independent Claims	2 - 3 =	0	x $ 84.00	$0.00	
☐ Multiple Dependent Claim(s) (if applicable)			+$280.00	$	
TOTAL OF ABOVE CALCULATIONS=				$890.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$	
SUBTOTAL=				$890.00	
Processing fee of **$130.00** for furnishing English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date.				$	
TOTAL NATIONAL FEE=				$890.00	
Fee for recording the enclosed assignment. The assignment must be accompanied by an appropriate cover sheet. **$40.00** per property +				$	
TOTAL FEE ENCLOSED=				$890.00	
				Amount to be. refunded	$
				charged	$

a. ☒ A check in the amount of $890.00 to cover the above fee is enclosed.

b. ☐ Please charge Deposit Account No. 12-1216 in the amount of $ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 12-1216. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Customer Number: 23460

23460

PATENT TRADEMARK OFFICE

Carol Larcher

Carol Larcher, Registration No. 35,243
One of the Attorneys for Applicant(s)

Date: January 8, 2002

U.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NO.
Unassigned 10/030466	PCT/EP00/06539	215110

CERTIFICATION UNDER 37 CFR 1.10

"Express Mail" Label Number: EL643546302US

Date of Deposit: January 8, 2002

I hereby certify that this express request to begin national examination procedures under 35 USC 371(f) of the International Patent Application referenced above, including all of the items listed thereon as enclosures, is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" Service under 37 CFR 1.10 on the date indicated above and is addressed to Box PCT, Commissioner for Patents, Attention: DO/EO/US, Washington, D.C. 20231.

Irina Mikitiouk

Printed Name of Person Signing:

I. Mikitiouk

Signature

PATENT
Attorney Docket No. 215110

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Feussner et al.

Art Unit: Unassigned

Application No. Unassigned
(U.S. National Phase of PCT/EP00/06539)

Examiner: Unassigned

Filed: January 8, 2002

For: 11-ARACHIDONATE-LIPOXYGENASE MUTANTS

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to the examination of the above-identified patent application, please enter the following amendments and consider the following remarks.

AMENDMENTS

IN THE SPECIFICATION:

Replace the paragraph beginning at page 1, line 6, with:
The present invention relates to a method for producing a plant lipoxygenase with modified positional specificity and to the lipoxygenase obtained by said method and to the use thereof for the hydroperoxylation of arachidonic acid at carbon atom 11.

Replace the paragraph beginning at page 2, line 28, with:
This problem is solved according to the invention by a method in which at least one amino acid is changed in a wild type LOX, preferably of potato tuber.

Replace the paragraph beginning at page 3, line 5, with:

Figure 3 shows the sequence of wild type LOX of potato tubers [SEQ ID NO:3]. The mutagenized amino acid position is underlined. Primers 1 and 2 [SEQ ID NOS: 1 and 2, respectively] as used are also shown.

Replace the paragraph beginning at page 3, line 8, with:
In a preferred embodiment, the amino acids are changed in the region of the amino acid position 570 to 581 of potato tuber LOX. The above-indicated amino acid positions refer to the sequence under the access number S73865 in the EMBL data base or the sequence according to Fig. 3. The positions in LOXs of other plant species, which correspond to the amino acid positions 593 to 602 of *Cucumis sativus* lipoxygenase, can easily be determined by sequence comparisons between sequence X92890 and the further protein sequences, e.g., of soybean, potato, arabidopsis, tobacco or barley. The following Table 1 shows the result of an amino acid comparison between the cucumber-derived enzyme and the corresponding positions in the enzymes of other plants. The first group (15-LOX) shows a comparison between LOXs which at position 15 introduce a hydroperoxy group into an arachidonic acid molecule, while the second group (5-LOX) shows a comparison between sequences which introduce a hydroperoxy group at position 5.

Replace the paragraph beginning at page 5, line 1, with:
The present invention further relates to LOX mutants which are obtainable according to the above-described methods. The LOXs according to the invention can be produced with the help of the methods known from the prior art, for example directed mutagenesis, and subsequent protein expression. In particular mutants which after incubation with arachidonic acid yield at least 40%, preferably 50%, of the derivative perhydroxylated at position 11 are considered to be inventive.

Replace the paragraph beginning at page 5, line 27, with:
Finally, new plants or plant parts can be regenerated from the above-mentioned cells by *in vitro* culturing methods. For the production of such transgenic plants the known transformation system can be used, e.g., on the basis of *Agrobacteria* and Ti plasmid derivatives.

Replace the paragraph beginning at page 6, line 4, with:
Particularly preferred is an arachidonic acid derivative which contains a hydroperoxy group at position 11. The derivative can then easily be converted into the hydroxy derivative. The thus available 11S-HPETE can be used for producing the alcohols, aldehydes and dicarbonic acids shown below. The enzyme hydroperoxide lyase is contained in extracts of cucumber seedlings, for example. 2E- and 3Z-nonenal and their alcohols are important flavorings in foodstuff (e.g., cucumbers).

Replace the paragraph beginning at page 7, line 15, with:
For bacterial expression of wild type LOX and LOX mutant and for directed mutagenesis, use was made of the plasmid pet3b (Novagen, Germany) which contained the cDNA of the potato tuber LOX as insert (pET-LOX1; cf. Geerts, A., Feltkamp, D., Rosahl, S. (1994) Expression of lipoxygenase in wounded tubers of *solanum tuberosum* L., Plant Physiol. 105: 269-277). Mutagenesis was carried out by using the QuikChange Mutagenesis Kit from Stratagene (Heidelberg, Germany). Oligonucleotides containing the appropriate base exchanges were purchased from MWG-Biotech (Ebersberg, Germany). To analyze the mutation, an additional conservative base exchange was introduced to construct a new restriction cleavage site. In addition, the mutation was sequenced and at least five different bacterial clones were expressed and used for analyzing the enzymatic characteristics. Expression of pET-LOX1 and its mutant was performed as described by Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436. Cells from 1 liter cultures were resuspended in 5-7 ml lysis buffer and disrupted by using a sonifier tip with pulses each of 30 seconds, and cellular debris was pelleted.

Replace the paragraph beginning at page 8, line 4, with:
For product analysis, 0.9 ml of cell lysates were incubated with 0.9 mM arachidonic acid (final concentration) in 100 mM Tris buffer, pH 7.5, for 30 minutes at room temperature. Reaction was stopped by the addition of sodium borohydride to convert the hydroperoxy fatty acids formed to the corresponding hydroxy compounds. The samples were acidified to pH 3 and the lipids were extracted (cf. Bligh, E.G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917). The lower chloroform phase was recovered and the solvent was evaporated. The remaining lipid was dissolved with 0.1 ml methanol, and aliquots were subjected to HPLC analysis.

Replace the paragraph beginning at page 8, line 15, with:
HPLC analysis was carried out on a Hewlett Packard 1100 HPLC system coupled to a diode detector. RP-HPLC of the free fatty acid derivatives was carried out on a Nucleosil C-18 column (Macherey-Nagel, 250 x 4 mm, 5µm particle size) with a solvent system of methanol/water/acetic acid (85/15/0.1; v/v/v) and at a flow rate of 1 ml/min. Absorption at 234 nm (absorption of the conjugated diene system of the hydroxy fatty acids) and at 210 nm (polyenoic fatty acids) was recorded accordingly. Straight-phase HPLC (SP-HPLC) of hydroxy fatty acid isomers was carried out on a Zorbax SIL column (HP, Waldbronn, Germany; 250 x 4.6 mm, 5 µm particle size) with a solvent system of n-hexane/2-propanol/acetic acid (100/2/0.1, v/v/v) at a flow rate of 1ml/min. The enantiomer composition of the hydroxy fatty acids was analyzed by chiral-phase HPLC on a Chiralcel OD column (Daicel Chem. Industries, distributed by Baker Chem., Deventer, Netherlands; 250 x 4.6 mm, 5µm particle size) with a solvent system of hexane/2-propanol/acetic acid (100/5/0.1, v/v/v) at a flow rate of 1 ml/min. (Cf. Feussner, I., Balkenhohl, T.J., Porzel, A., Kühn, H.& Wasternack, C. (1997) J. Biol. Chem. 272, 21635-21641).

Replace the paragraph beginning at page 9, line 10, with:
The starting cDNA and the mutagenesis kit were as described above. For analysis of the mutation further conservative base exchanges were carried out for producing a new restriction cleavage site for BsTBL. The following primers were used for producing the mutation V576F: GCT GGT GGG GTT CTT GAG AGT ACA TTC TTT CCT TCG AAA TTT GCC ATG GAA ATG TCA GCT G (coding strand) [SEQ ID NO:1] and CAG CGT ACA TTT CCA TGG CAA ATT TCG AAG GAA AGA ATG TAC TCT CAA GAA CCC CAC CAG C (complementary strand) [SEQ ID NO:2]. Furthermore, the mutant was sequenced and 5 different bacterial colonies were expressed and used for enzymatic studies. The expression of pET-LOX1 was carried out as described above. The further preparation was carried out as already indicated above. Analysis of the produced fatty acid derivative (containing a hydroperoxy group at position 11) was carried out as indicated above. The result of the SP-HPLC analysis for converting arachidonic acid with V576F is shown in Fig. 2. The following Table 2 shows a comparison of the specificity of the wild type (wtLOX) with the mutant ($LOXV_{576}F$).

IN THE CLAIMS:

Please cancel claims 1-11.

Please add the following new claims:

12. (New) A method of enhancing the specificity of a plant lipoxygenase for position 11 of arachidonic acid comprising changing at least one amino acid in a wild type plant lipoxygenase, characterized in that the change takes place at position 576 of potato tuber lipoxygenase or at a corresponding position in a lipoxygenase of another plant species, whereupon the specificity of the plant lipoxygenase for position 11 of arachidonic acid is enhanced.

13. (New) The method according to claim 12, characterized in that the change at position 576 results in the presence of a Phe residue at position 576.

14. (New) The method according to claim 12, characterized in that the amino acid change is effected by directed mutagenesis.

15. (New) The method according to claim 13, characterized in that the amino acid change is effected by directed mutagenesis.

16. (New) An isolated or purified lipoxygenase obtainable by the method of claim 12.

17. (New) An isolated or purified lipoxygenase obtainable by the method of claim 13.

18. (New) An isolated or purified nucleic acid encoding the lipoxygenase of claim 16.

19. (New) An isolated or purified nucleic acid encoding the lipoxygenase of claim 17.

20. (New) An isolated or purified vector comprising the nucleic acid of claim 18.

21. (New) An isolated or purified vector comprising the nucleic acid of claim 19.

22. (New) A cell comprising the nucleic acid of claim 18 and/or a vector comprising said nucleic acid.

23. (New) A cell comprising the nucleic acid of claim 19 and/or a vector comprising said nucleic acid.

24. (New) A plant or a plant part comprising the cell of claim 22.

25. (New) A plant or a plant part comprising the cell of claim 23.

26. (New) A method for producing 11-perhydroxy arachidonic acid or the reduced 11-hydroxy derivative thereof comprising incubating arachidonic acid with the lipoxygenase of claim 16 under appropriate conditions, whereupon 11-perhydroxy arachidonic acid is obtained, and, optionally, reducing the 11-perhydroxy arachidonic acid, whereupon the reduced 11-hydroxy derivative thereof is obtained.

27. (New) A method for producing 11-perhydroxy arachidonic acid or the reduced 11-hydroxy derivative thereof comprising incubating arachidonic acid with the lipoxygenase of claim 17 under appropriate conditions, whereupon 11-perhydroxy arachidonic acid is obtained, and, optionally, reducing the 11-perhydroxy arachidonic acid, whereupon the reduced 11-hydroxy derivative thereof is obtained.

28. (New) An arachidonic acid derivative containing a hydroxy group at position 11.

IN THE ABSTRACT:

Replace the Abstract with:

A method for producing a plant lipoxygenase (LOX) with modified positional specificity toward arachidonic acid and its use for hydroperoxylation of arachidonic acid. The LOX makes it possible to produce for the first time (11*S*,14*Z*,12*E*,8*Z*,5*Z*)-11-hydroperoxy-14,12,8,5-eicosatetraenic acids on a large scale. To this end, arachidonic acid is incubated as substrate with the LOX under appropriate conditions. Hydroperoxylation of the arachidonic acid is then effected, preferably at position 11, with secondary products which are hydroperoxylated at position 8, at position 5, or at positions 11 and 8 and 5.

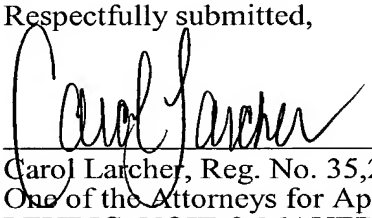
REMARKS

Conclusion

The present application is the U.S. national phase of a PCT application. The specification and the abstract have been amended to correct inadvertent typographical and translation errors and to insert SEQ ID NOs. In addition, claims 1-11 have been cancelled, and claims 12-28 have been added. The claims have been amended to conform the claims to U.S. patent practice and to eliminate multiple claim dependencies. No new matter has been added by way of these amendments.

The application is considered in good and proper form for allowance, and the Examiner is respectfully requested to pass this application to issue. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



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Date: January 8, 2002

PATENT
Attorney Docket No. 215110

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Feussner et al.

Art Unit: Unassigned

Application No. Unassigned
(U.S. National Phase of PCT/EP00/06539)

Examiner: Unassigned

Filed: January 8, 2002

For: 11-ARACHIDONATE-LIPOXYGENASE MUTANTS

PENDING CLAIMS AFTER ENTRY OF PRELIMINARY AMENDMENT

12. A method of enhancing the specificity of a plant lipoxygenase for position 11 of arachidonic acid comprising changing at least one amino acid in a wild type plant lipoxygenase, characterized in that the change takes place at position 576 of potato tuber lipoxygenase or at a corresponding position in a lipoxygenase of another plant species, whereupon the specificity of the plant lipoxygenase for position 11 of arachidonic acid is enhanced.

13. The method according to claim 12, characterized in that the change at position 576 results in the presence of a Phe residue at position 576.

14. The method according to claim 12, characterized in that the amino acid change is effected by directed mutagenesis.

15. The method according to claim 13, characterized in that the amino acid change is effected by directed mutagenesis.

16. An isolated or purified lipoxygenase obtainable by the method of claim 12.

17. An isolated or purified lipoxygenase obtainable by the method of claim 13.

18. An isolated or purified nucleic acid encoding the lipoxygenase of claim 16.

19. An isolated or purified nucleic acid encoding the lipoxygenase of claim 17.

20. An isolated or purified vector comprising the nucleic acid of claim 18.

21. An isolated or purified vector comprising the nucleic acid of claim 19.

22. A cell comprising the nucleic acid of claim 18 and/or a vector comprising said nucleic acid.

23. A cell comprising the nucleic acid of claim 19 and/or a vector comprising said nucleic acid.

24. A plant or a plant part comprising the cell of claim 22.

25. A plant or a plant part comprising the cell of claim 23.

26. A method for producing 11-perhydroxy arachidonic acid or the reduced 11-hydroxy derivative thereof comprising incubating arachidonic acid with the lipoxygenase of claim 16 under appropriate conditions, whereupon 11-perhydroxy arachidonic acid is obtained, and, optionally, reducing the 11-perhydroxy arachidonic acid, whereupon the reduced 11-hydroxy derivative thereof is obtained.

27. A method for producing 11-perhydroxy arachidonic acid or the reduced 11-hydroxy derivative thereof comprising incubating arachidonic acid with the lipoxygenase of claim 17 under appropriate conditions, whereupon 11-perhydroxy arachidonic acid is obtained, and, optionally, reducing the 11-perhydroxy arachidonic acid, whereupon the reduced 11-hydroxy derivative thereof is obtained.

28. An arachidonic acid derivative containing a hydroxy group at position 11.

PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Feussner et al.

Application No. Unassigned
(U.S. National Phase of PCT/EP00/06539)

Filed: January 8, 2002

For: 11-ARACHIDONATE-LIPOXYGENASE MUTANTS

Art Unit: Unassigned

Examiner: Unassigned

AMENDMENTS TO SPECIFICATION, CLAIMS, AND ABSTRACT MADE VIA PRELIMINARY AMENDMENT

Amendments to the paragraph beginning at page 1, line 6:
The present invention relates to a method for producing a plant lipoxygenase with modified positional specificity and to the lipoxygenase obtained by said method and to the use thereof for the hydroperoxylation of arachidonic acid [to] at carbon atom 11.

Amendments to the paragraph beginning at page 2, line 28:
This problem is solved according to the invention by a method in which at least one amino acid is [ex]changed in a wild type LOX, preferably of potato tuber.

Amendments to the paragraph beginning at page 3, line 5:
Figure 3 shows the sequence of wild type LOX of potato tubers [SEQ ID NO:3]. The mutagenized amino acid position is underlined. Primers 1 [und] and 2 [SEQ ID NOS: 1 and 2, respectively] as used are also shown.

Amendments to the paragraph beginning at page 3, line 8:
In a preferred embodiment, the amino acids are [ex]changed in the region of the amino acid position 570 to 581 of potato tuber LOX. The above-indicated amino acid positions refer to the sequence under the access number S73865 in the EMBL data base or the sequence according to Fig. 3. The positions in LOXs of other plant species, which correspond to the amino acid positions 593 to 602 of *Cucumis sativus* lipoxygenase, can easily be determined

by sequence comparisons between sequence X92890 and the further protein sequences, e.g., of soybean, potato, arabidopsis, tobacco or barley. The following Table 1 shows the result of an amino acid comparison between the cucumber-derived enzyme and the corresponding positions in the enzymes of other plants. The first group (15-LOX) shows a comparison between LOXs which at position 15 introduce a hydroperoxy group into an arachidonic acid molecule, while the second group (5-LOX) shows a comparison between sequences which introduce a hydroperoxy group at position 5.

Amendments to the paragraph beginning at page 5, line 1:
The present invention further relates to LOX mutants which are obtainable according to the above-described methods. The LOXs according to the invention can be produced with the help of the methods known from the prior art, for example directed mutagenesis, and subsequent protein expression. In particular mutants which after incubation with arachidonic acid yield at least 40%, preferably 50%, of the derivative perhydroxylated [to] at position 11 are considered to be inventive.

Amendments to the paragraph beginning at page 5, line 27:
Finally, new plants or plant parts can be regenerated from the above-mentioned cells by *in vitro* culturing methods. For the production of such transgenic plants the known transformation system can [e.g.] be used, e.g., on the basis of [*a*] *Agrobacteria* and Ti plasmid derivatives.

Amendments to the paragraph beginning at page 6, line 4:
Particularly preferred is an arachidonic acid derivative which contains a hydroperoxy group at position 11. The derivative can then easily be converted into the hydroxy derivative. The thus available 11S-HPETE can be used for producing the alcohols, aldehydes and dicarbonic acids shown below. The enzyme hydroperoxide lyase is [e.g.] contained in extracts of cucumber seedlings, for example. 2E- and 3Z-nonenal and their alcohols are important flavorings in foodstuff (e.g., cucumbers).

Amendments to the paragraph beginning at page 7, line 15:
For bacterial expression of wild type LOX and LOX mutant and for directed mutagenesis, use was made of the plasmid pet3b (Novagen, Germany) which contained the cDNA of the

potato tuber LOX as insert (pET-LOX1; cf. Geerts, A., Feltkamp, D., Rosahl, S. (1994) Expression of lipoxygenase in wounded tubers of *solanum tuberosum* L., Plant Physiol. 105: 269-277). Mutagenesis was carried out by using the QuikChange Mutagenesis Kit from Stratagene (Heidelberg, Germany). Oligonucleotides containing the appropriate base exchanges were purchased from MWG-Biotech (Ebersberg, Germany). To analyze the mutation, an additional conservative base exchange was introduced to construct a new restriction cleavage site. In addition, the mutation was sequenced and at least five different bacterial clones were expressed and used for analyzing the enzymatic characteristics. Expression of pET-LOX1 [und] and its mutant was performed as described by Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436. Cells from 1 liter cultures were resuspended in 5-7 ml lysis buffer and disrupted by using a sonifier tip with pulses each of 30 seconds, and cellular debris was pelleted.

Amendments to the paragraph beginning at page 8, line 4:
For product analysis, 0.9 ml of cell lysates [was] were incubated with 0.9 mM arachidonic acid (final concentration) in 100 mM Tris buffer, pH 7.5, for 30 minutes at room temperature. Reaction was stopped by the addition of sodium borohydride to convert the hydroperoxy fatty acids formed to the corresponding hydroxy compounds. The samples were acidified to pH 3 and the lipids were extracted (cf. Bligh, E.G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917). The lower chloroform phase was recovered and the solvent was evaporated. The remaining lipid was dissolved with 0.1 ml methanol, and aliquots were subjected to HPLC analysis.

Amendments to the paragraph beginning at page 8, line 15:
HPLC analysis was carried out on a Hewlett Packard 1100 HPLC system coupled to a diode detector. RP-HPLC of the free fatty acid derivatives was carried out on a Nucleosil C-18 column (Macherey-Nagel, 250 x 4 mm, 5µm particle size) with a solvent system of methanol/water/acetic acid (85/15/0.1; v/v/v) and at a flow rate of 1 ml/min. Absorption at 234 nm (absorption of the conjugated diene system of the hydroxy fatty acids) and at 210 nm (polyenoic fatty acids) [were] was recorded accordingly. Straight-phase HPLC (SP-HPLC) of hydroxy fatty acid isomers was carried out on a Zorbax SIL column (HP, Waldbronn, Germany; 250 x 4.6 mm, 5 µm particle size) with a solvent system of n-hexane/2-propanol/acetic acid (100/2/0.1, v/v/v) at a flow rate of 1ml/min. The enantiomer

composition of the hydroxy fatty acids was analyzed by chiral-phase HPLC on a Chiralcel OD column (Daicel Chem. Industries, distributed by Baker Chem., Deventer, Netherlands; 250 x 4.6 mm, 5µm particle size) with a solvent system of hexane/2-propanol/acetic acid (100/5/0.1, v/v/v) at a flow rate of 1 ml/min. (Cf. Feussner, I., Balkenhohl, T.J., Porzel, A., Kühn, H.& Wasternack, C. (1997) J. Biol. Chem. 272, 21635-21641).

Amendments to the paragraph beginning at page 9, line 10:
The starting cDNA and the mutagenesis kit were as described above. For analysis of the mutation further conservative base exchanges were carried out for producing a new restriction cleavage site for BsTBL. The following primers were used for producing the mutation V576F: GCT GGT GGG GTT CTT GAG AGT ACA TTC TTT CCT TCG AAA TTT GCC ATG GAA ATG TCA GCT G (coding strand) [SEQ ID NO:1] and CAG CGT ACA TTT CCA TGG CAA ATT TCG AAG GAA AGA ATG TAC TCT CAA GAA CCC CAC CAG C (complementary strand) [SEQ ID NO:2]. Furthermore, the mutant was sequenced and 5 different bacterial colonies were expressed and used for enzymatic studies. The expression of pET-LOX1 was carried out as described above. The further preparation was carried out as already indicated above. Analysis of the produced fatty acid derivative (containing a hydroperoxy group at position 11) was carried out as indicated above. The result of the SP-HPLC analysis for converting arachidonic acid with V576F is shown in Fig. 2. The following Table 2 shows a comparison of the specificity of the wild type (wtLOX) with the mutant ($LOXV_{576}F$).

Amendments to existing claims:

[1. A method of enhancing the specificity of a plant lipoxygenase for position 11 of arachidonic acid, comprising the step of

- exchanging at least one amino acid in a wild type lipoxygenase, characterized in that the exchange takes place at position 576 of potato tuber lipoxygenase or at a corresponding position in a lipoxygenase of another plant species.]

[2. The method according to claim 2, characterized in that the exchange at position 576 leads to the presence of a Phe residue in the mutant.]

[3. The method according to one of claims 1 or 2, characterized in that the amino acid exchange is effected by directed mutagenesis.]

[4. Lipoxygenase obtainable by a method according to any one of claims 1 to 3.]

[5. Nucleic acid coding for a lipoxygenase according to claim 4.]

[6. Vector containing a nucleic acid according to claim 5.]

[7. Cell containing a nucleic acid according to claim 5 and/or a vector according to claim 6.]

[8. Plant or plant part comprising a host cell according to claim 7.]

[9. A method for producing 11-perhydroxy arachidonic acid or the reduced 11-hydroxy derivative, comprising the step of

- converting arachidonic acid with a lipoxygenase according to claim 6 and, optionally, reducing the perhydroxy compound obtained to hydroxy compound.]

[10. Use of a lipoxygenase according to claim 4 for producing 11-perhydroxy arachidonic acid and/or 11-hydroxy arachidonic acid.]

[11. Arachidonic acid derivative containing a hydroxy group at position 11.]

12. A method of enhancing the specificity of a plant lipoxygenase for position 11 of arachidonic acid comprising changing at least one amino acid in a wild type plant lipoxygenase, characterized in that the change takes place at position 576 of potato tuber lipoxygenase or at a corresponding position in a lipoxygenase of another plant species, whereupon the specificity of the plant lipoxygenase for position 11 of arachidonic acid is enhanced.

13. The method according to claim 12, characterized in that the change at position 576 results in the presence of a Phe residue at position 576.

14. The method according to claim 12, characterized in that the amino acid change is effected by directed mutagenesis.

15. The method according to claim 13, characterized in that the amino acid change is effected by directed mutagenesis.

16. An isolated or purified lipoxygenase obtainable by the method of claim 12.

17. An isolated or purified lipoxygenase obtainable by the method of claim 13.

18. An isolated or purified nucleic acid encoding the lipoxygenase of claim 16.

19. An isolated or purified nucleic acid encoding the lipoxygenase of claim 17.

20. An isolated or purified vector comprising the nucleic acid of claim 18.

21. An isolated or purified vector comprising the nucleic acid of claim 19.

22. A cell comprising the nucleic acid of claim 18 and/or a vector comprising said nucleic acid.

23. A cell comprising the nucleic acid of claim 19 and/or a vector comprising said nucleic acid.

24. A plant or a plant part comprising the cell of claim 22.

25. A plant or a plant part comprising the cell of claim 23.

26. A method for producing 11-perhydroxy arachidonic acid or the reduced 11-hydroxy derivative thereof comprising incubating arachidonic acid with the lipoxygenase of claim 16 under appropriate conditions, whereupon 11-perhydroxy arachidonic acid is obtained, and, optionally, reducing the 11-perhydroxy arachidonic acid, whereupon the reduced 11-hydroxy derivative thereof is obtained.

27. A method for producing 11-perhydroxy arachidonic acid or the reduced 11-hydroxy derivative thereof comprising incubating arachidonic acid with the lipoxygenase of claim 17 under appropriate conditions, whereupon 11-perhydroxy arachidonic acid is obtained,

and, optionally, reducing the 11-perhydroxy arachidonic acid, whereupon the reduced 11-hydroxy derivative thereof is obtained.

28. An arachidonic acid derivative containing a hydroxy group at position 11.

Amendments to the abstract:

[The present invention relates to a] A method for producing a plant lipoxygenase (LOX) with modified positional specificity toward arachidonic acid and [to] its use for hydroperoxylation of arachidonic acid. [In particular, t] The [inventive] LOX makes it possible to produce for the first time (11*S*,14*Z*,12*E*,8*Z*,5*Z*)-11-hydroperoxy-14,12,8,5-eicosatetraenic acids [at] on a large scale. To this end, arachidonic acid is incubated as substrate with the [inventive] LOX under appropriate conditions. Hydroperoxylation of the arachidonic acid is then effected, preferably at position 11, with secondary products which are hydroperoxylated at position 8, at [or] position 5, or at positions 11 and 8 and 5.

11-ARACHIDONATE-LIPOXYGENASE MUTANTS

The present invention relates to a method for producing a plant lipoxygenase with modified positional specificity and to the lipoxygenase obtained by said method and to the use thereof for the hydroperoxylation of arachidonic acid to carbon atom 11.

Lipoxygenases (LOXs, linolic acid: oxygen oxidoreductase; EC.1.13.11.12; LOXs) are widely distributed in the plant and animal kingdom (Siedow, J.N. (1991) Ann. Rev. Plant Physiol. Plant Mol. Biol. 42, 145-188; Yamamoto, S. (1992) Biochim. Biophys. Acta 1128, 117-131). These enzymes constitute a family of iron-containing dioxygenases that catalyze a region- (or position-) and stereo-selective oxygenation of polyenoic fatty acids to hydroperoxy derivatives (Rosahl, S. (1996) Z. Naturforsch. 51c, 123-138). In mammals, LOXs are classified according to their specificity for specific positions during arachidonic acid oxygenation (Yamamoto, S. (1992) Biochim. Biophys. Acta 1128, 117-131; Schewe, T., Rapaport, S.M. & Kühn, H. (1986) Adv. Enzymol. Mol. Biol 58, 191-272). 15-, 12-, 8- and 5-LOXs have so far been isolated here. LOXs which effect the insertion of oxygen at positions 9 and 11, respectively, on the carbon skeleton of arachidonic acid have not been known yet (Yamamoto, S. (1992) Biochim. Biophys. Acta 1128, 117-131). Since arachidonic acid either is not present in higher plants or is only present in small amounts as a constituent of storage lipids, plant LOXs are classified as 9- and 13-LOXs. This nomenclature is derived from the position at which oxygenation takes place in linolic acid (LA) (Gardner, H.W. (1991) Biochim. Biophys. Acta 1084, 221-239). Recently, a more comprehensive classification of plant LOXs has been proposed based on a comparison of the primary structures (Shibata, D. & Axelrod, B. (1995) J. Lipid Mediators Cell Signal. 12, 213-228). The specificity of a LOX for a specific position is the result of two catalytic partial reactions:

(i)

Regio- and stereospecific hydrogen removal; in fatty acids containing several double bonds (such as linolenic acid, arachidonic acid, or eicosapentaenoic acid), hydrogen removal is possible at various positions.

(ii)

Regio- and stereospecific oxygen insertion (oxygen can be inserted at various positions (either at the +2 or –2 position)) (cf. Fig. 1). Thus, a fatty acid containing three double-allylic methylenes, such as arachidonic acid, can be oxygenated by a LOX to six regioisomeric hydroperoxy derivatives (HPETEs), namely 15- and 11-HPETE (these originate from the removal of hydrogen at position C-13), 12- and 8-HPETE (these originate from hydrogen removal at position C-10) and 9- and 5-HPETE (these originate from hydrogen removal at position C-7). Experiments on mammalian 12- and 15-LOXs indicated that the position of hydrogen removal can be altered when critical amino acids are changed by directed mutagenesis (Borngräber, S., Kuban, R. J., Anton, M. & Kühn, H. (1996) J. Mol. Biol. 264, 1145-1153; Sloane, D.L., Leung, R., Craik, C. S. & Sigal, E (1991) Nature 354, 149-152). Attempts to alter the LOX reactivity from a +2 to a –2 rearrangement or vice versa (e.g., conversion of a linoleate 13-LOX to a 9-LOX) by directed mutagenesis have recently been successful (Hornung, E., Walther, M., Kühn, H. & Feussner, I. (1999) Proc. Natl. Acad. Sci. USA 96, 4192-4197).

It has been the object of the present invention to indicate a method for providing a LOX with a desired C-11 positional specificity in arachidonic acid.

This problem is solved according to the invention by a method in which at least one amino acid is exchanged in a wild type LOX, preferably of potato tuber.

Figure 1 shows the specificity of a LOX reaction with substrates containing two allylic methylenes.

Figure 2 shows the HPLC analysis of hydroxy fatty acids obtained with the help of wild type LOX of potato tubers and the V576F mutant of arachidonic acid after reduction of hydroperoxy fatty acids with sodium borohydride.

Figure 3 shows the sequence of wild type LOX of potato tubers. The mutagenized amino acid position is underlined. Primers 1 und 2 as used are also shown.

In a preferred embodiment, the amino acids are exchanged in the region of the amino acid position 570 to 581 of potato tuber LOX. The above-indicated amino acid positions refer to the sequence under the access number S73865 in the EMBL data base or the sequence according to Fig. 3. The positions in LOXs of other plant species, which correspond to the amino acid positions 593 to 602 of *Cucumis sativus* lipoxygenase, can easily be determined by sequence comparisons between sequence X92890 and the further protein sequences, e.g., of soybean, potato, arabidopsis, tobacco or barley. The following Table 1 shows the result of an amino acid comparison between the cucumber-derived enzyme and the corresponding positions in the enzymes of other plants. The first group (15-LOX) shows a comparison between LOXs which at position 15 introduce a hydroperoxy group into an arachidonic acid molecule, while the second group (5-LOX) shows a comparison between sequences which introduce a hydroperoxy group at position 5.

Table 1

Comparison of amino acid residues involved in the specificity of a plant LOX for a specific position (15 or 5).

ENZYME	Access No.	Position of amino acid residue	Amino acid residue
15-LOX			
Cucumber lipid body LOX	X92890	596/597	Thr/His
Soybean seed LOX-1	P08170	556/557	Thr/Phe
Potato LOX-H1	X96405	614/615	Ser/Phe
Arabidopsis LOX-2	P38418	611/612	Cys/Phe
5-LOX			
Potato LOX	S73865	575/576	Thr/Val
Tobacco elicitor-induced LOX	X84040	580/581	Thr/Val
Barley grain LOX-A	L35931	574/575	Thr/Val

The sequence motif at position 570 to 581 is GVLESTVFPSK (sequence according to S73865).

In a particularly preferred embodiment the exchange takes place at position 576 of sequence S73865. At position 576, there is a Val residue in the wild type. The residue at position 576 is here replaced by a Phe residue. The exchange in the region of the amino acid position 570 to 581 has the effect that the potato tuber 5-LOX is converted to an arachidonic acid 11-LOX. In the following this mutant will also be designated as V576F. The wild type sequence is shown as Fig. 3. Position 576 is marked.

Preferably, the amino acids are exchanged in the wild type with the help of directed mutagenesis, as is sufficiently known in the prior art.

The present invention further relates to LOX mutants which are obtainable according to the above-described methods. The LOXs according to the invention can be produced with the help of the methods known from the prior art, for example directed mutagenesis, and subsequent protein expression. In particular mutants which after incubation with arachidonic acid yield at least 40%, preferably 50%, of the derivative perhydroxylated to position 11 are considered to be inventive.

The present invention further relates to nucleic acids which code for the LOXs according to the invention. Starting from the wild type sequences available in the prior art, the sequences according to the invention can be produced by directed mutagenesis.

Furthermore, the present invention relates to vectors into which the nucleic acids according to the invention are introduced for the purpose of cloning and expression. Corresponding cloning and expression vectors are sufficiently known to the skilled artisan from the prior art (cf. Maniatis et al. Molecular Cloning, A Laboratory Manual (1989), Cold Spring Hator Laboratory Press).

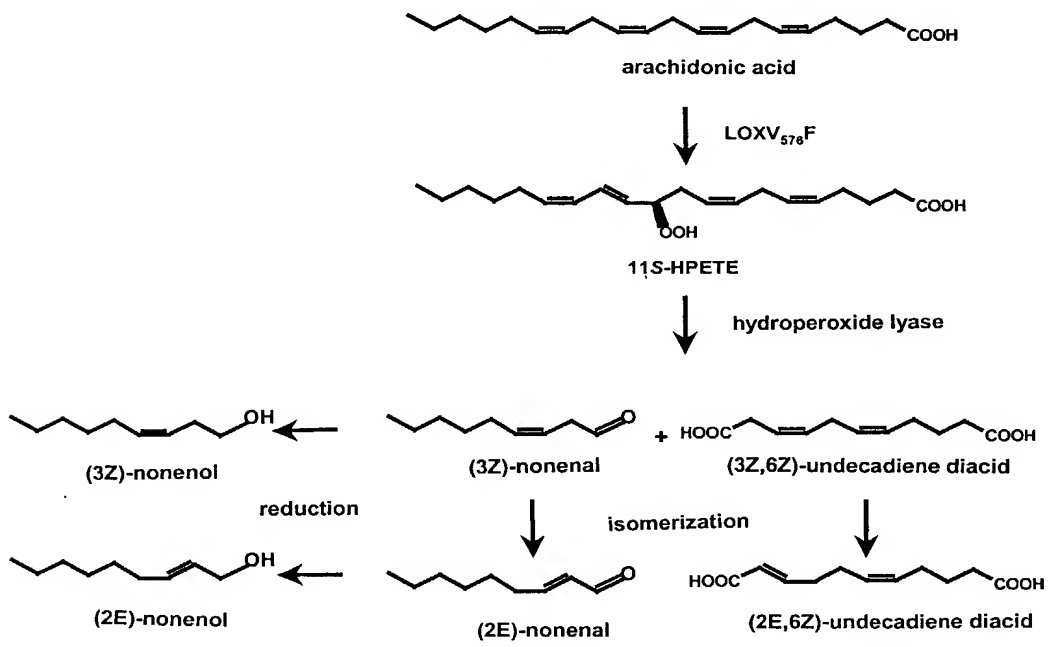
The present invention further relates to a cell into which the nucleic acid according to the invention or the vector according to the invention is introduced. After introduction of the nucleic acid or the vector, the cell is then capable of expressing a LOX for the first time or at a large scale. The fatty acid pattern of a cell can thereby be changed in a targeted way, with the result that the phenotype of the cell can be altered in different respects. This includes, inter alia, a different composition of the cell membrane.

Finally, new plants or plant parts can be regenerated from the above-mentioned cells by *in vitro* culturing methods. For the production of such transgenic plants the known transformation system can e.g. be used on the basis of *agrobacteria* and Ti plasmid derivatives.

The LOXs according to the invention make it possible to produce for the first time new arachidonic acid derivatives at a large scale. To this end, arachidonic acid is incubated as a substrate with the LOXs according to the invention under appropriate

conditions. Hydroperoxylation of the arachidonic acid is then effected, preferably at position 11.

Particularly preferred is an arachidonic acid derivative which contains a hydroperoxy group at position 11. The derivative can then easily be converted into the hydroxy derivative. The thus available 11S-HPETE can be used for producing the alcohols, aldehydes and dicarbonic acids shown below. The enzyme hydroperoxide lyase is e.g. contained in extracts of cucumber seedlings. 2E- and 3Z-nonenal and their alcohols are important flavorings in foodstuff (e.g. cucumbers).



Such an arachidonic acid derivative has so far not been available because a LOX of an appropriate positional specificity has been missing.

The further examples serve to explain the invention.

1. Preparation of the mutant V576F

Materials:

The chemicals used were obtained from the following sources: standards of chiral and racemic hydroxy fatty acids were obtained from Chayman Chem (Ann Arbor, Mi, USA). Methanol, hexane, 2-propanol (all HPLC grade) were obtained from Baker (Griesheim, Germany). Restriction enzymes were purchased from New England BioLabs (Schwalbach, Germany).

Directed mutagenesis and protein expression:

For bacterial expression of wild type LOX and LOX mutant and for directed mutagenesis, use was made of the plasmid pet3b (Novagen, Germany) which contained the cDNA of the potato tuber LOX as insert (pET-LOX1; cf. Geerts, A., Feltkamp, D., Rosahl, S. (1994) Expression of lipoxygenase in wounded tubers of *solanum tuberosum* L. Plant Physiol. 105: 269-277). Mutagenesis was carried out by using the QuikChange Mutagenesis Kit from Stratagene (Heidelberg, Germany). Oligonucleotides containing the appropriate base exchanges were purchased from MWG-Biotech (Ebersberg, Germany). To analyze the mutation, an additional conservative base exchange was introduced to construct a new restriction cleavage site. In addition, the mutation was sequenced and at least five different bacterial clones were expressed and used for analyzing the enzymatic characteristics. Expression of pET-LOX1 und its mutant was performed as described by Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436. Cells from 1 liter cultures were resuspended in 5-7 ml lysis buffer and disrupted by using a sonifier tip with pulses each of 30 seconds, and cellular debris was pelleted.

Activity assay and sample preparation:

For product analysis, 0.9 ml of cell lysates was incubated with 0.9 mM arachidonic acid (final concentration) in 100 mM Tris buffer, pH 7.5, for 30 minutes at room temperature. Reaction was stopped by the addition of sodium borohydride to convert the hydroperoxy fatty acids formed to the corresponding hydroxy compounds. The samples were acidified to pH 3 and the lipids were extracted (cf. Bligh, E.G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917). The lower chloroform phase was recovered and the solvent was evaporated. The remaining lipid was dissolved with 0.1 ml methanol, and aliquots were subjected to HPLC analysis.

Analysis:

HPLC analysis was carried out on a Hewlett Packard 1100 HPLC system coupled to a diode detector. RP-HPLC of the free fatty acid derivatives was carried out on a Nucleosil C-18 column (Macherey-Nagel, 250 x 4 mm, 5µm particle size) with a solvent system of methanol/water/acetic acid (85/15/0.1; v/v/v) and at a flow rate of 1 ml/min. Absorption at 234 nm (absorption of the conjugated diene system of the hydroxy fatty acids) and at 210 nm (polyenoic fatty acids) were recorded accordingly. Straight-phase HPLC (SP-HPLC) of hydroxy fatty acid isomers was carried out on a Zorbax SIL column (HP, Waldbronn, Germany; 250 x 4.6 mm, 5 µm particle size) with a solvent system of n-hexane/2-propanol/acetic acid (100/2/0.1, v/v/v) at a flow rate of 1ml/min. The enantiomer composition of the hydroxy fatty acids was analyzed by chiral-phase HPLC on a Chiralcel OD column (Daicel Chem. Industries, distributed by Baker Chem., Deventer, Netherlands; 250 x 4.6 mm, 5µm particle size) with a solvent system of hexane/2-propanol/acetic acid (100/5/0.1, v/v/v) at a flow rate of 1 ml/min. (Cf. Feussner, I., Balkenhohl, T.J., Porzel, A., Kühn, H.& Wasternack, C. (1997) J. Biol. Chem. 272, 21635-21641).

2. Preparation of the LOX-V576F mutant:

The reagents and methods employed for preparing said mutant were substantially as already described above. A few modifications of the above-mentioned methods which were specifically adapted to the preparation of the V576F mutant are now explained.

Directed mutagenesis and protein expression:

The starting cDNA and the mutagenesis kit were as described above. For analysis of the mutation further conservative base exchanges were carried out for producing a new restriction cleavage site for BsTBL. The following primers were used for producing the mutation V576F: GCT GGT GGG GTT CTT GAG AGT ACA TTC TTT CCT TCG AAA TTT GCC ATG GAA ATG TCA GCT G (coding strand) and CAG CGT ACA TTT CCA TGG CAA ATT TCG AAG GAA AGA ATG TAC TCT CAA GAA CCC CAC CAG C (complementary strand). Furthermore, the mutant was sequenced and 5 different bacterial colonies were expressed and used for enzymatic studies. The expression of pET-LOX1 was carried out as described above. The further preparation was carried out as already indicated above. Analysis of the produced fatty acid derivative (containing a hydroperoxy group at position 11) was carried out as indicated above. The result of the SP-HPLC analysis for converting arachidonic acid with V576F is shown in Fig. 2. The following Table 2 shows a comparison of the specificity of the wild type (wtLOX) with the mutant ($LOXV_{576}F$).

Table 2

Comparison of the product specificity of wtLOX and LOXV_{576}F with arachidonic acid

Enzyme	(15S,13*E*,11*Z*,8*Z*,5*Z*)-20:4	(12S, 14*Z*, 10*E*,8*Z*,5*Z*)-20:4	(11S,14*Z*,12*E*,8*Z*, 5*Z*)-20:4	(9S,14*Z*,11*Z*,7*E*, 5*Z*)-20:4	(8S,14*Z*,11*Z*9*E*, 5*Z*)-20:4	(5*S*, 14*Z*, 11*Z*,8*Z*6*E*)-20:4
wtLOX	5 %	6 %	23 %	3 %	21 %	42 %
LOXV_{576}F	9 %	4 %	50 %	3 %	23 %	11 %

3. Description of the figures

Figure 1 shows that the positional specificity of the LOX reaction depends on the site of hydrogen cleavage and the orientation of the radical. The [+2] radical arrangement indicates that oxygen is inserted at the second carbon atom in the direction of the methyl terminus of the substrate counted from the site of hydrogen removal. [-2] indicates the inverse orientation of the radical arrangement.

Figure 2 shows HPLC analysis of fatty acids with the mutant V576F. Equal amounts of LOX protein were incubated with 0.9 mM arachidonic acid at room temperature for 30 minutes. After reduction of lipids with sodium borohydride, the reaction mixture was acidified to pH 3, and the lipids were extracted. Oxygenated fatty acid derivatives were isolated by RP-HPLC, and the individual positional isomers were analyzed by SP-HPLC. Ratios of S and R were determined by HPLC (insets).

Figure 3 shows the amino acid sequence of the wild type lipoxygenase of potato tubers. The mutated Val576 is underlined.

Abbreviations used:

CP-HPLC	for	chiral-phase HPLC;
RP-HPLC	for	reverse-phase HPLC;
SP-HPLC	for	straight-phase HPLC;
HPETE	for	hydroperoxy arachidonic acid;
LOX	for	lipoxygenase;
HETE	for	hydroxy arachidonic acid

Article 34

International Patent Application PCT/EP00/06539
INSTITUT FÜR PFLANZENBIOCHEMIE – IPB et al.
Ref.:PCT1200-03138/GRI
Date: October 2, 2001

1. A method of enhancing the specificity of a plant lipoxygenase for position 11 of arachidonic acid, comprising the step of
   - exchanging at least one amino acid in a wild type lipoxygenase, characterized in that the exchange takes place at position 576 of potato tuber lipoxygenase or at a corresponding position in a lipoxygenase of another plant species.

2. The method according to claim 2, characterized in that the exchange at position 576 leads to the presence of a Phe residue in the mutant.

3. The method according to one of claims 1 or 2, characterized in that the amino acid exchange is effected by directed mutagenesis.

4. Lipoxygenase obtainable by a method according to any one of claims 1 to 3.

5. Nucleic acid coding for a lipoxygenase according to claim 4.

6. Vector containing a nucleic acid according to claim 5.

7. Cell containing a nucleic acid according to claim 5 and/or a vector according to claim 6.

8. Plant or plant part comprising a host cell according to claim 7.

9. A method for producing 11-perhydroxy arachidonic acid or the reduced 11-hydroxy derivative, comprising the step of

- converting arachidonic acid with a lipoxygenase according to claim 6 and, optionally, reducing the perhydroxy compound obtained to hydroxy compound.

10. Use of a lipoxygenase according to claim 4 for producing 11-perhydroxy arachidonic acid and/or 11-hydroxy arachidonic acid.

11. Arachidonic acid derivative containing a hydroxy group at position 11.

Abstract

The present invention relates to a method for producing a plant lipoxygenase with modified positional specificity toward arachidonic acid and to its use for hydroperoxylation of arachidonic acid. In particular, the inventive LOX makes it possible to produce for the first time (11*S*,14*Z*,12*E*,8*Z*,5*Z*)-11-hydroperoxy-14,12,8,5-eicosatetraenic acids at a large scale. To this end, arachidonic acid is incubated as substrate with the inventive LOX under appropriate conditions. Hydroperoxylation of the arachidonic acid is then effected, preferably at position 11, with secondary products which are hydroperoxylated at position 8 or position 5 or at position 11 and 8 and 5.

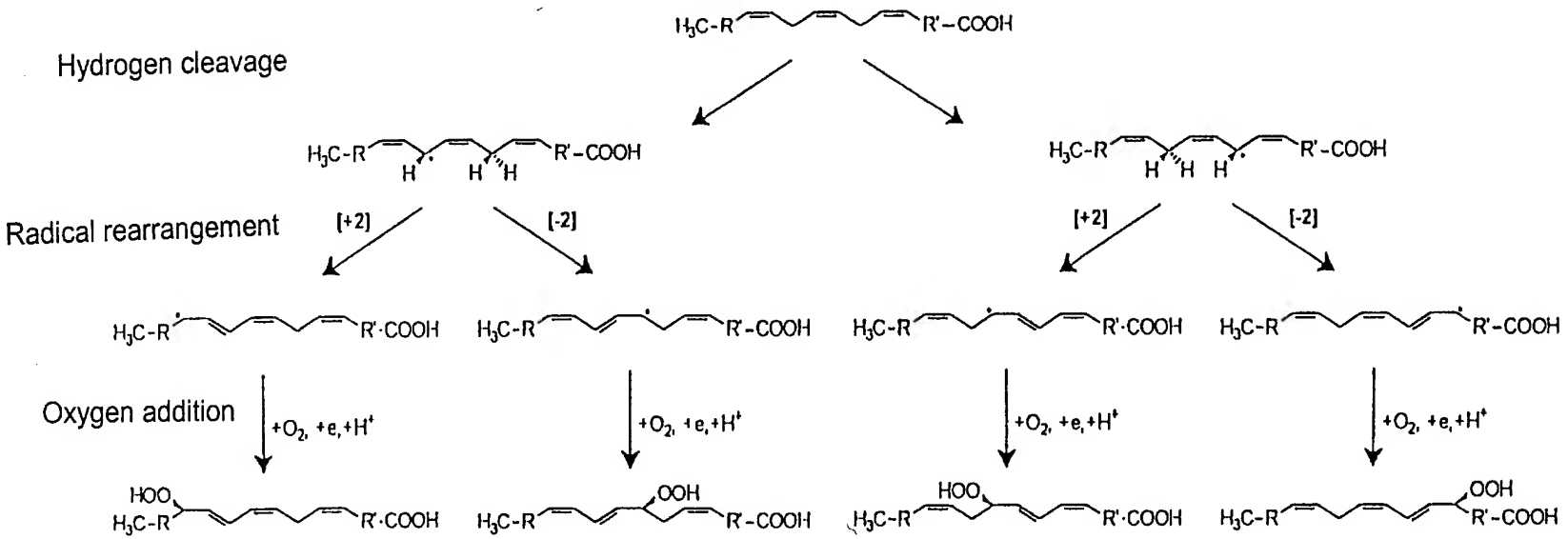


Fig. 1

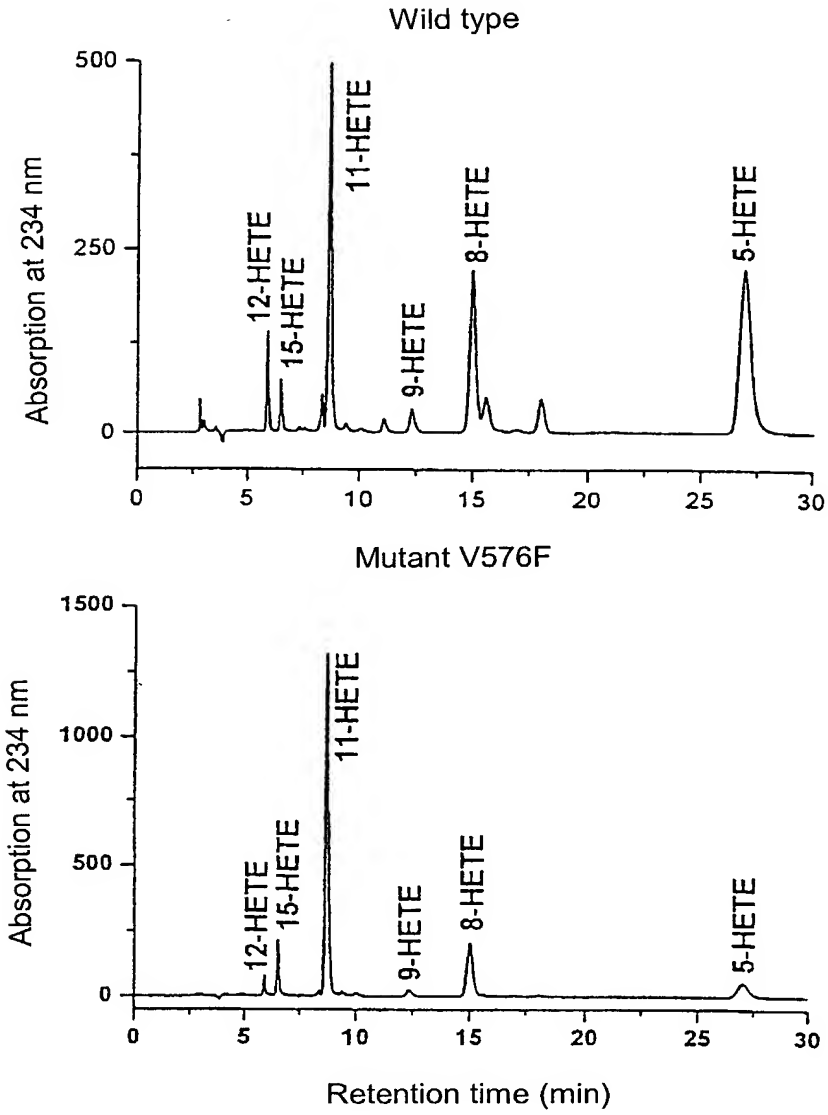

Wild type
Absorption at 234 nm
500
250
0
12-HETE
15-HETE
11-HETE
9-HETE
8-HETE
5-HETE
0
5
10
15
20
25
30
Mutant V576F
Absorption at 234 nm
1500
1000
500
0
12-HETE
15-HETE
11-HETE
9-HETE
8-HETE
5-HETE
0
5
10
15
20
25
30
Retention time (min)

Fig. 2

1 QIVGGLIGGH HDSKKVKGTV VMMKKNALDF TDLAGSLTDK IFEALGQKVS FQLISSVQS
D
61 PANGLQGKHS NPAYLENFLF TLTPLAAGET AFGVTFDWNE EFGVPGAFII KNTHINEFF
L
121 KSLTLEDVPN HGKVHFVCNS WVYPSFRYKS DRIFFANQPY LPSETPELLR KYRENELLT
L
181 RGDGTGKREA WDRIYDYDVY NDLGNPDQGE QNVRTTLGGS ADYPYPRRGR TGRPPTRTD
P
241 KSESRIPLIL SLDIYVPRDE RFGHLKMSDF LTYALKSIVQ FILPELHALF DGTPNEFDS
F
301 EDVLRLYEGG IKLPQGPLFK ALTAAIPLEM MKELLRTDGE GILRFPTPLV IKDSKTAWR
T
361 DEEFAREMLA GVNPIIISRL QEFPPKSKLD PEAYGNQNST ITAEHIEDKL DGLTVDEAM
N
421 NNKLFILNHH DVLIPYLRRI NTTTTKTYAS RTLLFLQDNG SLKPLAIELS LPHPDGDQF
G
481 VISKVYTPSD QGVESSIWQL AKAYVAVNDS GVHQLISHWL NTHAVIEPFV IATNRQLSV
L
541 HPIHKLLYPH FRDTMNINAM ARQILINAGG VLESTVFPSK FAMEMSAVVY KDWVFPDQA
L
601 PADLVKRGVA VEDSSSPHGV RLLIEDYPYA VDGLEIWSAI KSWVTDYCSF YYGSDEEIL
K
661 DNELQAWWKE LREVGHGDKK NEPWWPEMET PQELIDSCTT IIWIASALHA AVNFGQYPY
A
721 GYLPNRPTVS RRFMPEPGTP EYEELKKNPD KAFLKTITAQ LQTLLGVSLI EILSRHTTD
E
781 IYLGQRESPE WTKDKEPLAA FDKFGKKLTD IEKQIIQRNG DNILTNRSGP VNAPYTLLF
P
841 TSEGGLTGKG IPNSVSI

Primer1: GCT GGT GGG GTT CTT GAG AGT ACA TTC TTT CCT TCG AAA TTT GCC ATG GAA ATG TCA GCT G

Primer2: CAG CGT ACA TTT CCA TGG CAA ATT TCG AAG GAA AGA ATG TAC TCT CAA GAA CCC CAC CAG C

Fig. 3

PATENT
Attorney Docket No. 215110

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

This declaration is of the following type:

☐ original ☐ design ☐ supplemental
☒ national stage of PCT
☐ divisional ☐ continuation ☐ continuation-in-part

My residence, post office address, and citizenship are as stated below next to my name. I believe I am the original, first, and sole inventor *(if only one name is listed below)* or an original, first, and joint inventor *(if plural names are listed below)* of the subject matter which is claimed and for which a patent is sought on the invention entitled:

11-ARACHIDONATE LIPOXYGENASE MUTANTS

the specification of which:

☐ is attached hereto.
☒ was filed on January 8, 2002 as Application No. 10/030,464 and was amended on January 8, 2002 *(if applicable)*.
☐ was filed by Express Mail No. *as Application No. not known yet*, and was amended on *(if applicable)*.
☐ was filed on as PCT International Application No. PCT/ and was amended on *(if any)*.

I state that I have reviewed and understand the contents of the specification identified above, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to the patentability of the application identified above in accordance with 37 CFR 1.56.

I claim foreign priority benefits under 35 USC 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent, utility model, design registration, or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter and having a filing date before that of the application(s) from which the benefit of priority is claimed.

PRIOR FOREIGN PATENT, UTILITY MODEL, AND DESIGN REGISTRATION APPLICATIONS						
COUNTRY	PRIOR FOREIGN APPLICATION NO.	DATE OF FILING (day,month,year)	PRIORITY CLAIMED			
Germany	199 31 819.0	08 July 1999	X	YES		NO
				YES		NO
				YES		NO

I claim the benefit pursuant to 35 USC 119(e) of the following United States provisional patent application(s):

PRIOR U.S. PROVISIONAL PATENT APPLICATIONS, BENEFIT CLAIMED UNDER 35 USC 119(e)	
APPLICATION NO.	DATE OF FILING (day,month,year)

I claim the benefit pursuant to 35 USC 120 of any United States patent application(s) or PCT international application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this patent application is not disclosed in the prior patent application(s) in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose material information as defined in 37 CFR 1.56 effective between the filing date of the prior patent application(s) and the national or PCT international filing date of this patent application.

PRIOR U.S. PATENT APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S., BENEFIT CLAIMED UNDER 35 USC 120					
U.S. PATENT APPLICATIONS			Status *(check one)*		
U.S. APPLICATION NO.	U.S. FILING DATE		PATENTED	PENDING	ABANDONED
1.					
2.					
3.					
PCT APPLICATIONS DESIGNATING THE U.S.			Status *(check one)*		
PCT APPLICATION NO.	PCT FILING DATE (day,month,year)	U.S. APPLICATION NOS. ASSIGNED (if any)	PATENTED	PENDING	ABANDONED
4. PCT/EP00/06539	10 July 2000			X	
5.					
6.					

DETAILS OF FOREIGN APPLICATIONS FROM WHICH PRIORITY CLAIMED UNDER 35 USC 119 FOR ABOVE LISTED U.S./PCT APPLICATIONS				
ABOVE APPLICATION. NO.	COUNTRY	APPLICATION NO.	DATE OF FILING (day,month,year)	DATE OF ISSUE (day,month,year)
1.				
2.				
3.				
4. PCT/EP00/06539	Germany	199 31 819.0	08 July 1999	
5.				
6.				

In re Appln. of Feussner et al.
Attorney Docket No. 215110

As a named inventor, I hereby appoint Leydig, Voit & Mayer, Ltd. to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Customer Number 23460.

I further direct that correspondence concerning this application be directed to Leydig, Voit & Mayer, Ltd.: Customer Number 23460.

I declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's signature Ivo Feussner
Dr. Ivo Feussner

Date April 5, 2002

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In re Appln. of Feussner et al.
Attorney Docket No. 215110

2-20

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In re Appln. of Feussner et al.
Attorney Docket No. 215110

3rd

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SEQUENCE PROTOCOL

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